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PRODUCTION OF AXENIC VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI IN ASSOCIATION WITH ROOT ORGAN CULTURE AND EFFICACY TEST BY FIELD TRIAL

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ABSTRACT

The present experiment was conducted during the year of 2014-15 at Patanjali Bio Research Institute, Uttarakhand, India. The objective of this research was to develop processes for production of contaminant free vesicular arbuscular mycorrhizal (VAM) fungal inoculum through In-vitro method by using Zea mays as hosts for the fungus and to investigate its effectiveness as Biofetilizer formulation on Zea mays as test crop grown under field condition. The viable spores were isolated from Cynodon dactylon roots and its rhizosphere soil and analyzed for native association with VAM fungi. The isolated viable spores were co-cultured with Zea mays roots developed through plant tissue culture. The VAM biofertilizer was formulated by mixing the axenic VAM spores with bentonite carrier and tested for efficiency in the field. The finding of this experiment revealed that the colonization percentage (91.28±1.63), number of vesicles (38.47±0.93), viability of spore (89.9±2.09%) and spore density (4459.0±401.49) were significantly high for the VAM inoculum developed through root culture in comparison to conventional method (VAM inoculum production by pot culture, on-farm, aeroponic system, hydroponic cultures & nutrient film technique). The efficacy of developed product was tested by using Anola fruit compost (two type fine and course) with VAM fertilizer and Farm Yard Manure (FYM) with VAM fertilizers whereas control received only FYM. The field trial result showed that vegetative growth and fruit yield were significantly higher in treatments received VAM fertilizer than untreated control. Most of the traits were reported significantly higher in treatment T2 which received VAM fertilizer with coarse compost. The parameters like plant fresh weight (159.27±23.80 g), plant dry weight (52.81±7.14 g), root fresh weight (34.24±5.65 g), root dry weight (10.84±2.06 g), root numbers/plant (38.67±1.65), root length (30.22±0.81 cm), leaf surface area (328.89±52.25 cm^2), fruit yield (41.73±4.89 quintal/acre), number of fruits/acre (22.93±2.43 $nX10^3$), fruit weight (233.81±18.46 g), fruit length (31.0±1.39 cm), number of grains/fuit (446.78±6.73), fresh grain weight (121.06±8.69 g), dry grain weight (62.39±7.25 g) and fruit girth (16.78±0.49 cm) were recorded significantly higher for the treatment T2 having VAM fertilizer with course compost. This is the first kind of experiment where plant tissue culture technique was applied for the formulation of axenic VAM biofertilizers as well as tested for its efficacy at field level.

KEYWORDS: Vesicular Arbuscular Mycorrhiza, Axenic Spore Production, Product Formulation, Efficacy Test

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Original Article

INTRODUCTION

Microbes are considers a boon for agricultural sustainability because they involved in nutrient transformation process, decomposed resistant components of plant and animal tissue, play role in microbial antagonism, participate in humus formation, improves soil structure, acts as plant growth promoters and performs numerous metabolic pathways (Rocheli de Souza et al-2015). The potential agents for these activities are

rhizosphere-competent fungi and bacteria. Mycorrhizal fungi are one of the soil microbes, which form mutualistic relationships with roots of 90% of plants (Quilambo, O.A. 2003). The association between Mycorrhiza and plants is believed to be as old as plant (Pirozynski, K.A. & Malloch D.W. 1975). These mycorrhizal fungi require plants to complete their life cycle and in turn stimulate absorption of nutrients and water, control plant diseases, and improve soil structure and so arbuscular mycorrhizal fertilizers are considered as an alternative to costly chemical input (Simard S.W., Perry D.A. & Jones M.D. 1997; Finlay R.D., Lindahl B.D. & Taylor A.F.S. 2008; Srivastava D. et al. 1996; JN Larsen J & Jakobsen I. 2008). A lot of evidences have accumulated in the recent past, about how effectively the use of the symbiotic microbes could contribute, in decreasing use of fertilizer and pesticide in agriculture, forestry and flori-hotriculture. Although the importance of mycorrhiza for agriculture and horticulture has been recognized since long, its application has been limited due to its uncultivable nature. The matter of concern for development of mycorrhiza based fertilizer is that, the growth of arbuscular mycorrhizae in pure culture depends upon the presence of living host roots, since it could not be cultivated on artificial nutrient medium like other microbes. On the other hand, if produced by soil based medium like in pot or field, pure spore will not obtained due to cross contamination with other soil/air born microbes. Some other alternative culture techniques like airoponics, hydroponic and plants tissue culture could be promising to produces pure Vesicular Arbuscular Mycorrhizal (VAM) spores. The present study was conducted to mass production of VAM inoculum by applying the technique of Plant Tissue Culture, VAM based biofertilizer formulation and to assets the efficacy on maize crops at field level. The ultimate objective of laboratory and field studies is to extend them to the field on a commercial level.

MATERIALS AND METHODS

Study Site and Sample Collection

This study was conducted during the year of 2014-15 located at Patanjali Bio Research Institute (PBRI), Padartha, Haridwar, Uttarakhand, India. Rhizosphere soil along with root of *Cynodon dactylon* collected from the garden of Ptanjali Bio Research Institute. 5 healthy plants were selected randomly from the study site. The roots of the plant and the rhizosphere soil were taken as the sample. The soil (100 g) sample was collected at 0-50 cm depth. The root samples were washed thoroughly in tap water. The study site for field efficacy test was Patanajli Bio Research Institute research field located at Patanjali Food and Herbal Park, village Padartha, Haridwar, Uttarakhand, India.

Analysis for Native Association, Spore Isolation and Viability Test

Biermann and Liadermann (1981) method was applied to find the Mycorrhizal association with the root. Roots were chopped up to 1 cm size and stained as per method developed by Phillips and Hayman (1970). Roots were thoroughly washed with tap water and soften by adding 5% KOH solution, heated at 80 ^{o}C for 5-15 minutes and then carefully washed 4-5 times with purified water. Soft roots were dipped in 1% HCl solution for 4-5 minutes and then dipped in Trypan blue for overnight. De-staining of the acidified roots was done by Lactoglycerol for 8 hours and then association (VAM mycelia, vesicles, arbusculars, and spores) was conform by microscopy. Total 20 segments of size 0.5 to 1.0 cm of root from each sample were analyzed by mounting at glass slide with lactophenol. The percentage of colonization was calculated by using the formula given below

$$\% \text{ of Colonization} = \frac{\text{Total number of VAM positive segment x 100}}{\text{Total number of root segment observed}}$$

To Isolate the VAM spores from rhizosphare soil, wet sieving and decanting method of Gerdemann and Nicolson

(1963) were applied. 100 g dried soil sample was dissolve in 250 ml water and the suspension leave for 1 hour with shaking at regular interval. The suspension was passed through the a series of sieves of size 250 micron, 100 micron and 53 micron arranged in descending order. Roots and coarse debris were collected on 250 micron sieve, while spores were collected on subsequent sieves according to their size. Residue from each sieve was collected in petri dish along with water. The spores were observed under microscope and count was expressed as spores/g of the sample. Spores identifications such as spore size, colour, spore wall, hyphal attachment was based on the method of Schenck and Prez (1990). The viability of spores was determined by the method of National Centre of Organic Farming, Ghaziabad, India. Freshly collected spores were dipped into 0.25% staining solution of MTT (2,5-diphenyl-2N-tetrazolium bromide) and incubated at 27 ^{o}C in dark. The spores were observed under microscope at 24 hours, 48 hours and 72 hours of incubation. The spores which retain red/pink colour were treated as viable and percentage of viability was calculated using the following formula

$$\% \text{ of Viability} = \frac{\text{No. of spores which stained red or pink} \times 100}{\text{Total number of spores}}$$

Co-Culture with Maize Host under Aseptic Condition (PTC), Harvesting of Colonized Roots and Spores

The dual culture of mychorrhiza and *Zea mays* (host) root were established by using plant tissue culture technique. Maize seeds were sterilized with 0.1 % mercuric chloride solution for 5 minutes under Laminar Air Flow, rinsed 3 times with autoclaved distilled water, inoculated in tubes on Whatman No.1 filter paper soaked under ¼ strength of MS liquid medium with pH 5.8 (Murashige and Skoog, 1962). The inoculated tubes were incubated under dark at 24±2 ^{o}C for 5-7 days in order to facilate the germination of germ tube (Becard and Piche 1989). Rooted plants were transferred on sterilized semi solid MS medium with pH 5.8, supplemented with 2 ppm IAA, which was pre inoculated with 100 micro liter sterilized VAM spores (*Glomus intraradices and G. versiforme*) solution (Figure 2 A & B) having at least 200 spore/ml (The VAM spores isolated from the soil were sterilized by 0.1 % mercuric chloride and washed repeatedly with sterile distilled water in 53 micron sieve under aseptic condition)**.** The rooted plant was taken for VAM association because a study conducted by Prasad SP and Sulochana KK, Kerala Agricultural University, India show that rooted plant best suited for AM inoculation. The association was observed by the method described above at every 7 days interval of inoculation.

The 8 weeks old cultures were harvested by using the in-house method developed by Patanjali Bio Research Institute, Haridwar, India. The Culture medium was cut into big chunks and de-ionized by adding 1:1 volume of 10 mM sodium citrate buffer with pH-6.0 (Citrate buffer was prepared by dissolving 21.01 gm Citric acid in 1000 ml distilled water and 29.41 gm tri-Sodium Acetate in 1000 ml distilled water separately then 9.5 ml of citric acid solution was mixed with 41.5 ml of tri-Sodium Citrate to make the pH 6.0). The de-ionized media was dissolve by putting the flask on shaker with rotation speed 150 per minute. The dissolve media along with cut roots were pour on stacks of sieves of pore size ranging from 250, 100 and 53 micron and washed gently with sterile water to remove the trace of media and buffer. The inoculums (Roots and spore along with trace of water) retain on sieves were transferred to dry butter paper and dry under hot air oven at 50 ^{o}C until constant weight. The dried inoculum was grind to powder of particle size 2000-2500 micron size.

VAM Product Formulation

The dried grind power was mixed with the carrier (bentonite) to make a product with at least 100 VAM spores per gram of the product. Bentonite was chosen as carrier because its water holding capacity is in the range of 2-15% due to which the spore can be stored in sterile bentonite for up to 4 years at 5^{o}C. Kremer, R.J. & Peterson, H.L. (1983); Paczkowski, M.W. & Berryhill, D.L. (1979) reported that bentonite as good as peat as carrier for biofertilizers formulation. Bentonite shows a good adhesive property after mixed with a little amount of water, which is the prime requirement for a good carrier. Bentonite provides large surface area by dispersing into colloidal particle and hence function as a carrier medium (Arthur G. Clem & Robert W. Doehler). To formulate the biofetilizer, 25 mg of dried powder was mixed with one kg of bentonite , and the number of spores in the developed product was ensure by the method of Gerdemann and Nicolson (1963).

Experimental Crop

Maize (*Zea mays*) crop was selected for both in-vitro spore production and efficacy test at field level field. The plant was selected because of the quick growing nature and short life. It is well known that this plant form a good association with VAM. Also the root analysis and staining is easier in this plant

Efficacy Test of developed product by field experiment

The product (VAM fertilizer) developed by the mixing of bentonite with grind power (obtained by in-vitro culture) was tested for efficacy through field experiment. The experiment was carried out during Kharif season, July, 2015 at the Patanjali Bio Research Institute, Padratha, Haridwar, India. This experiment was conducted with use of split plot as randomized block design with six treatments including one control each with three replications. The treatments were composed of To: FYM-200 kg/acre, T1: Aonla fruit (*Emblica officinalis* G.) waste fine compost 200 kg/acre (mixed with 1 inch thick bed surface soil) + VAM fertilizer 10 kg/acre, T2: Aonla fruit waste coarse compost 200 kg/acre + VAM fertilizer 10 kg/acre, T3: FYM 200 kg/acre + VAM fertilizer 5 kg/acre and T4: FYM 200 kg/acre + VAM fertilizer 10 kg/acre. The bed size was 6 m^2 (1 m x 6 m). 58 seeds of *Zea mays* were sown in each bed with seeds to seeds space 20 cm and row to row space 60 cm. The seeds were treated with Trichoderma sp. for 20 minutes before sowing. Intermediate treatment with same basal dose was applied at 20 DAS (Days After Sowing). Different morphological parameters like Plant height, Plant fresh and dry biomass, Shoot diameter, Numbers of node plant^{-1}, Numbers of leaf plant^{-1}, Leaf surface area, Root number plant^{-1}, Root fresh and dry weight, Numbers of fruits plant^{-1}, Numbers of grains fruit^{-1}, Fruit and Grain weight plant^{-1} and Total yield of fruits/grain treatments$^{-1.}$ Also the roots were analyzed to confirm the VAM association as per method described above. The morphological parameters were taken at two intervals, first at 35 DAS and the second at 90 DAS (i.e. on maturity). Total 180 plants were taken as sample for data collection. The shoot length was recorded in cm and considered from 0.5 cm above the soil to last node. Plants were separated into roots, stems, leaves and fruits. Leaf area (LA) plant^{-1} was measured by taking length and breadth (cm) of longest and shortest leaf with measuring scale. The fresh and dry weight (g) of whole plants (with root and leaves) and individual vegetative parts (roots, stems and leaves) were recorded. Fresh weight of shoots and roots were measured immediately and dry parameters were measured by drying the samples in an oven at 60 ^{o}C for constant weight. To record the roots parameters, roots were rinsed with tap water until all visible sand and soil particles were removed and excess water removed with blotting paper. Roots number plant^{-1} and root length (cm) also measured. Fresh and dry weight was weighed on a laboratory scale (Mettler AE 163, Switzerland). Fruits were harvested at the time of final harvest (90DAS) and the different parameters like Fruit number acre^{-1}, fruit weight

Quintal) acre^{-1}, average fruit length (cm) tretatment^{-1}, average fruit weight (g) tretatment$^{-1,}$ average number of grain fruit^{-1}, fresh and dry grain weight (g) fruit^{-1}, average fruit girth (cm) tretatment^{-1}. Fruit number and weight was recorded per plot and converted into per acre.

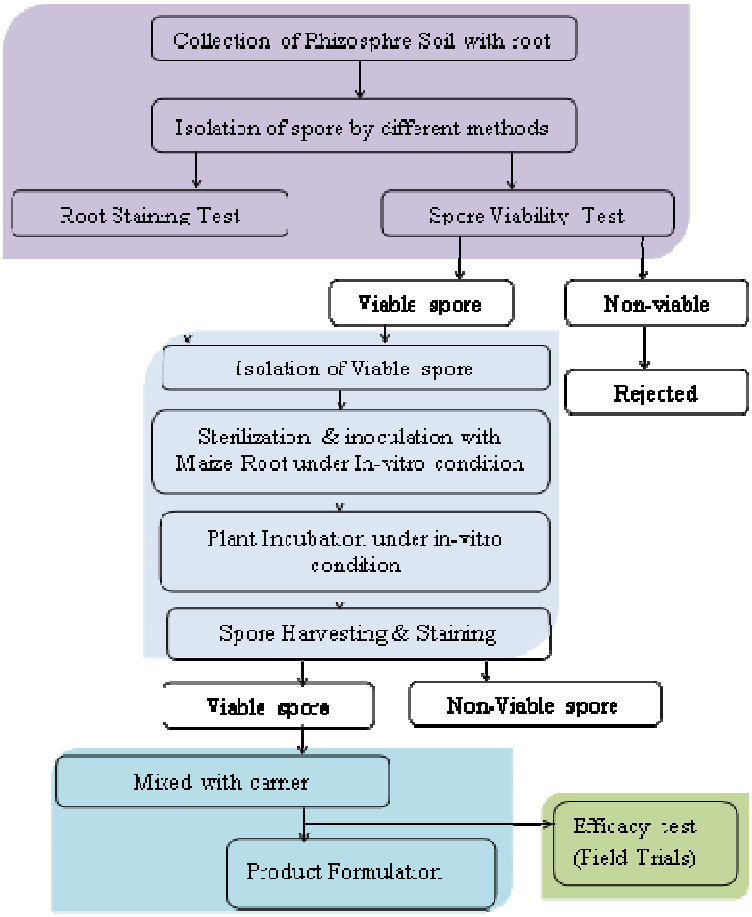

Figure 1: Process Flow Chart

Statistical Analysis

The data obtained in the experiment was subjected to analysis of variance (ANOVA). The software used was SPSS windows version 10 (SPSS Inc., Chicago, USA). Mean comparison was done using Tukey HSD comparison at 1% & 5% probability level.

RESULT AND DISCUSSIONS

The average mycelia root colonization, root vesicles, spores viability percentage and the average spore density in soil during primary screening was very poor as presented in table 1 (i.e. Rhizosphere soil along with root of *Cynodon dactylon* collected from the garden of Ptanjali Bio Research Institute to magnify with plant tissue culture technique). In *Cynodon dactylon* root colonization percentage was 25.71±0.45 and an average of 4.77±0.17 vesicles were observed in 1 cm of root bit. The viability percentage found 54.00±1.15 and the average of mycorrhizal spore density was found only 16.68±0.55 in 100 g of dry rhizosphere soil in the collected plant species *Cynodon dactylon.* The same parameter was very high in case of *Zea mays* root and the grind powder produced through plant tissue culture technique (Table 1). The root colonization percentage was 91.28±1.63 and an average of 38.47 ± .93 vesicles were observed in 1 cm of root bit.

The average of mycorrhizal spore density was found 4459.00±401.49 in 100 g of grind root powder of *Zea mays*. The spores obtained through In-vitro methods were bigger in size in comparison to rhizosphere spores and retain on 300 µ sieve. Also the viability percentage was very high and found 89.9±2.09 (Figure 2 C to H).

Table 1: Different Parameters in Native and in-Vitro Condition

Plant	Colonization Percentage	Number of Vesicles/1 cm Root	Viability Percentage	Average of Mycorrhizal Spore Density Per 100 g Soil/Root Powder
Cynodon dactylon (Native)	25.71±0.45	4.77±0.17	54.00±1.15	16.68±0.55
Zea mays (In-vitro)	91.28±1.63	38.47 ± .93	89.9±2.09	4459.00±401.49

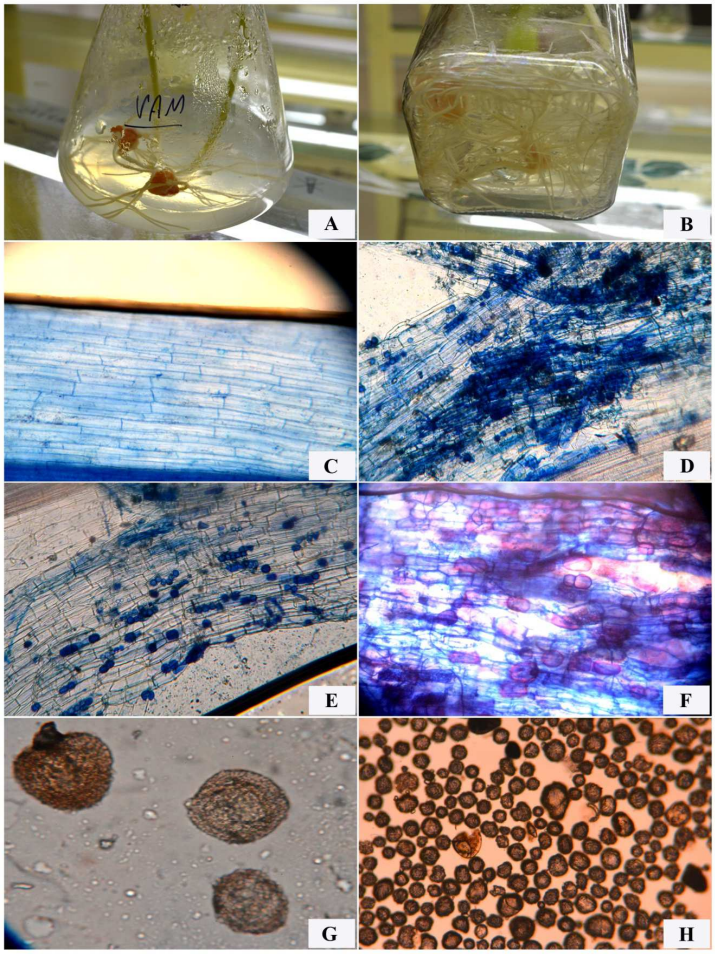

Figure 2: (a)(b): Axenic Culture Maize Root inoculated with VAM Spores, (c) Control without Infection, (d) VAM Hyphal Mass in Roots of Maize, (e) Newly Formed Vesicles and Spores, (f) Viable Vesicles and Spores Stained Pink Color, (g) Isolated Mature Spores and (h) Mature End/Ecto Spores and Vesicles. Image C-F and H were taken at 40x and Image G at 100x

The data presented in table 2 showed no any significant difference (at $P<0.05$) among treatments for any traits during 35 DAS but at 90 DAS most of the traits were found significantly higher ($p < 0.01$ or 0.05) for the all treatments having VAM with respect to untreated control. At 35 DAS the maximum plant height was recorded in T4 (73.83±2.03 cm) followed by T1 (73.67±8.09 cm) whereas it was 61.00±7.16 cm for control. Also highest number of leaves per plant was

recorded for T4. Leaf length (40.33±5.53 cm), Plant fresh weight (70.66±19.33 g), Root fresh weight (7.62±2.06 g) and Root dry weight (1.80±0.48 g) were observed highest for treatment T1 in comparison to control (35.33±3.17 cm, 52.29±12.52 g, 6.58±1.79 g and 1.45±0.36 g respectively). Plant dry weight was found maximum (14.45±7.96 g) for T2.

Values are Mean ± SE. Means in each row followed by the different letter significantly differs (P<0.05) from each other.

Table: 2: Different Morphological Traits at The 35 DAS

Morphological Trait	Treatment				
	T1	T2	T3	T4	T0
Plant Height	73.67±5.49a	65.00±3.86a	63.67±4.26a	73.83±1.70a	61.00±7.16a
Number of leaf per plant	7.83±0.75a	7.00±0.26a	7.17±0.60a	8.50±0.43a	6.50±1.06a
Leaf length(cm)	40.33±4.18a	34.83±2.65a	34.17±2.10a	35.83±1.38a	35.33±3.17a
Plant fresh weight(g)	70.66±13.95a	51.63±10.18a	46.32±9.38a	69.38±7.32a	52.29±12.52a
Plant Dry weight(g)	10.56±2.27a	14.45±7.96a	6.43±1.19a	8.34±0.70a	6.71±1.51a
Root fresh weight(g)	7.62±1.52a	5.30±1.31a	4.78±0.99a	6.93±1.08a	6.58±1.79a
Root Dry weight(g)	1.80±0.33a	1.13±0.34a	1.08±0.21a	1.30±0.24a	1.45±0.36a

The data obtained during the time of maturity (presented in table 3) showed that most of the morphological traits (15 out of 19) were recorded maximum for the treatment T2 (The treatment which received Enola waste course decomposed 200 kg /acre with 10 kg/acre VAM biofertilizers formulation) . This might be due the application of coarse decomposed manure application because coarse substances provides larger surface area and permit free gas exchange which helps better root development and rapid colonization as well as better growth condition to VAM spore (Okiobé S T et al 2016). Shoot height was found significantly higher (p<0.01) for treatment T1 (203.11±5.47 cm) in comparison to other treatments and control (167.55±11.13 cm). The plant fresh and dry weight was recorded maximum for T2 (159.27±23.80 g and 52.81±7.14 g respectively) whereas it was observed least for control (118.28±22.03 g and 33.82±6.3 g respectively). Also all roots traits were found higher for treatment T2. The root fresh weight for T2 was 34.24±5.65 g, root dry weight 10.84±2.06 g and root length 30.22±0.81 cm in comparison to 17.34±3.85 g, 6.91±1.44 g and 24.44±1.34 cm respectively for control. Root length for T2 was significantly higher (p<0.01) than control as well as other treatments. Number of roots plant^{-1} found significantly higher (at p<0.01) for treatment T2 (38.67±1.65) and T1 (36.67±2.48) with respect to other treatment and found minimum (25.11±3.10) for control. This result was obtained because the features of coarse decomposed manure as stated above. The shoot diameter (6.44±0.44 cm) and number of nodes/plant 12.33±0.62 were observed maximum for T1 whereas it was minimum in case of untreated control (5.11±0.51 cm and 10.78±0.40 respectively). Leaf surface area was recorded significantly higher (p<0.01) for treatment T2 (328.89±52.25 cm^2) whereas number of leaves plant^{-1} found maximum (9.89±0.82) for T1. Total fruit yield (quintal acre-^{1}) for treatment T1, T2, T3, T4 and control was 31.81±2.51, 41.73±4.89, 23.17±3.49, 30.23±7.38 and 19.69±3.07 respectively and found significantly higher for treatment T2 at p<0.05 (Table 3). Also total number of fruits acre^{-1} was recorded significantly higher (p<0.05) for treatment T2 and observed data (n x10^3) were 17.54±0.78, 22.93±2.43, 17.08±3.36, 15.74±0.23, 12.36±2.65 for T1, T2, T3, T4 and T0 respectively. The average fruit length was found significantly (p<0.01) higher for all treatments in comparison to control and recorded maximum for T2 (31.00±1.39 cm) whereas it was found 28.78±0.80cm, 26.89±0.29 cm, 27.78±0.62 cm and 19.00±0.58 cm for T1, T3, T4 and T0 respectively. The plants treated with T2 also had the significant (p<0.01) highest increase in average fruit weight 233.81±18.46 g followed by 204.34±11.67 g, 211.50±25.76 g and 193.79±20.93 g for T1, T4 and T3 respectively whereas it was recorded lowest (114.24±2.94 g) for control. The average number of grains fruit^{-}1 were found significantly higher

for all treatment with comparison to control but recorded maximum (446.78±6.73) for T2 followed by T4 (430.67±20.09), T1 (423.56±17.49), T3 (398.78±31.86) whereas lowest number (247.00±6.31) was observed for control. Fresh and Dry grains weight showed no any significant difference among treatments, although, recorded maximum for T2 (fresh weight 121.06±8.69 g and dry weight 62.39±7.25 g). Average fruit girth was significantly ($p<0.01$) higher for treatments T1, T2 and T4 with T3 and control. The observed fruit girth for T1, T2, T3, T4 and T0 was 16.55±0.40g, 16.78±0.49g, 15.89±0.40 g, 16.33±0.88 g and12.67±0.33 g respectively and found maximum for T2 (Figure 3 A to D).

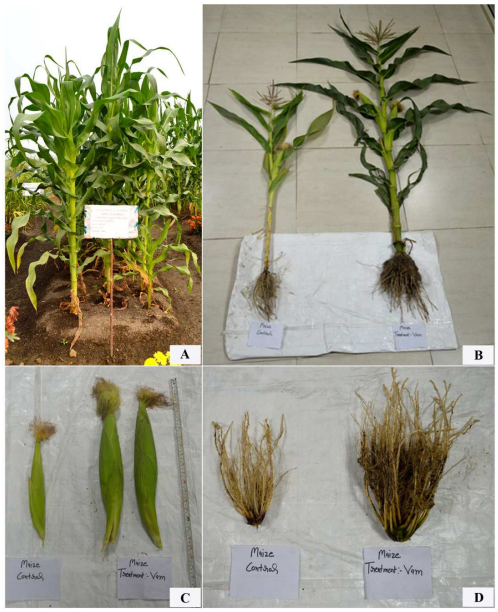

Figure 3: (A) Maize Crop Treated with Developed VAM Fertilizer Under Field, (B) Comparative Image of Height of Non-Treated and Treated Plant, (C) Comparative Image Showing Fruit Size Deference of Non-Treated And Treated Plant and (D) Comparative Image Showing Difference in Root Growth of Non-Treated and Treated Plant

Values are Mean ± SE. Means in each row followed by the different letter significantly differs ($P<0.01$* or $P<0.05$) from each other.

Table 3: Different Morphological Traits at the 90 DAS

Morphological Trait	Treatment				
	T1	T2	T3	T4	T0
Shoot height (cm)	203.11±5.47a*	192.88±7.38b	192.00C4.87b	181.44±3.94b	167.55±11.13b
Average Plant fresh weight (g)	158.51±26.86a	159.27±23.80a	117.22±15.13a	134.77±13.43a	118.28±22.03a
Average Plant dry weight (g)	51.23±7.21a	52.81±7.14a	33.26±4.06a	36.28±3.69a	33.82±6.3a
Average Root fresh weight plant-^{1} (g)	29.02±6.10a	34.24±5.65a	20.89±2.54a	21.76±3.24a	17.34±3.85a
Average Root dry weight/plant (g)	10.22±1.78a	10.84±2.06a	7.80±1.04a	8.13±1.38a	6.91±1.44a
Average Root length (cm)	27.89±1.17b	30.22±0.81a*	28.22±1.0b	28.56±0.78b	24.44±1.34b
Average Shoot diameter (cm)	6.44±0.44a	6.33±0.47a	6.00±0.37a	5.78±0.22a	5.11±0.51a

Table 3: Contd.,

Number of Nodes plant^{-1}	12.33±0.62a	11.78±0.40b	12.11±0.39b	10.56±0.24b	10.78±0.40b
Number of leaves plant^{-1}	9.89±0.82a	8.33±0.37a	9.00±0.67a	7.78±0.36a	8.00±0.33a
Number of roots plant^{-1}	36.67±2.48a*	38.67±1.65b*	32.67±1.89c	29.78±1.59c	25.11±3.10c
Leaves surface area (cm^2)	248.00±23.78b	328.89±52.25a	263.11±23.89b	293.44±43.03b	168.00±16.41b
Total yield (quintal acre^{-1})	31.81±2.51b	41.73±4.89a	23.17±3.49b	30.23±7.38b	19.69±3.07b
Total number of fruit acre^{-1} (nX10^3)	17.54±0.78b	22.93±2.43a	17.08±3.36b	15.74±0.23b	12.36±2.65b
Average fruit length (cm)	28.78±0.80a*	31.00±1.39b*	26.89±0.29c*	27.78±0.62d*	19.00±0.58e
Average fruit weight (g)	204.34±11.67a	233.81±18.46b*	193.79±20.93c	211.50±25.76d	114.24±2.94c
Average number of grains fruit^{-1}	423.56±17.49a*	446.78±6.73b*	398.78±31.86c*	430.67±20.09d*	247.00±6.31e
Average fresh grain weight fruit^{-1} (g)	103.26±5.16a	121.06±8.69a	99.40±11.76a	119.36±8.77a	101.53±4.07a
Average dry grain weight fruit^{-1}(g)	54.83±4.05a	62.39±7.25a	51.30±7.70a	61.49±3.61a	50.72±2.63a
Average fruit girth (cm)	16.55±0.40a*	16.78±0.49b*	15.89±0.40c	16.33±0.88d*	12.67±0.33e

Few efforts have been made in recent years to integrate the Plant Tissue Culture technique with Vesicular Arbuscular Mycorrhizal technique to obtain the axenic VAM fungus spores. Diop (1995) established a bank of germplasms of AM monoxenically cultivated in association with isolated tomato or transformed carrot roots A study conducted by Sawsan, A.A. & Abdel Rahman, R.A. (2012) revealed that Root Organ Culture support extensive root colonization with the formation of many arbuscular, vesicles and viable spores. Srinivasan, M. et al (2014) observed 8500-9000 spores per petri dish through transformed hairy carrot root culture further support the axenic production of VAM inoculum through PTC technique. Our research revealed that 91.28 % of inoculated root were colonized which is higher than the finding (86 %) of Nishi Mathur and Anil Vyas (2007) and (75-80%) of Srinivasan, M. et al (2014) and Jolicoeur M. et al (1998) reported 60 % viability of harvest spores whereas it was observed 89.9 % in the present study. Also our study reported 34.47 numbers of vesicles in 1 cm roots segments which was comparatively higher than the native/conventional method 18.20 as reported by Thomas, S. et al in 2014.

The VAM fertilizer effect on growth and yield attribute has been reported in numbers of crops. A lot of field trial was conducted in the previous years to analyze the effect of VAM fertilizer on morphological traits and yield attributes and revealed that VAM treated crops show better growth and yield. Our research finding confirming results of previous studies made by different scholar. Confirming results of Gautam Shrestha et al (2009), our research revealed that total plant height difference was observed significantly higher in VAM fertilizer applied treatments (203.11 cm) than untreated control (167.55 cm). Fruit yield and number of grains were found highest in VAM treated crops conforming the result of Sajedi, N. & Madani, H. (2006) and Hajilou, M.et al (2010) respectively. Also similar observations were made by Elwan (2001).

M.T. Vidal et. al. (1992) reported improved shoot and root growth in micropropagated Avocado plantlets supplemented with Mycorrhiza inoculum, our finding also revealed the same result.

CONCLUSIONS

From this experiment it is concluded that, the in-vitro methods provides extensive monoexnic viable spores (4459.0) which is much more than what previously reported by many authors. Also the inoculant (Biofertilizer) produced by this type (In-vitro) of VAM fungi significantly influence the morphological traits and greatly influencing the yield of agricultural and horticulture crops.

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